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14. ABSTRACT
The activity of the androgen receptor (AR) is critical for normal prostate development and function, but AR activity also plays a major role in the development and progression of prostate cancer. To better understand the function of beta-catenin in AR mediated transcription, we have identified a novel chromatin remodeling enzyme, CHD8, that can associate with beta-catenin and functions in AR mediated gene transcription. Year 1 was focused on the first specific aim of this proposal; the interaction of CHD8 with beta-catenin and the androgen receptor. Experiments were performed to characterize this interaction in vitro using recombinant proteins, and reagents were made to characterize this interaction in vivo using chromatin immunoprecipitation techniques. This work is required for the study of the function of CHD8 and β-catenin in AR mediated transcription and the ultimate understanding of the role played by CHD8 in prostate cancer progression.

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INTRODUCTION

The activity of the androgen receptor (AR) is critical for normal prostate development and function, but AR activity also plays a major role in the development and progression of prostate cancer. Current therapies utilize this fact and are aimed at reducing serum androgens and therefore, inhibiting AR. While these therapies show initial success in preventing tumor growth, they can ultimately fail due to the development of an androgen independent disease. β-catenin, a known oncogene, has been shown to directly interact with AR and also to function as a transcriptional coactivator for AR. To better understand the function of β-catenin in AR mediated transcription, we have identified a novel chromatin remodeling enzyme, CHD8, that can associate with β-catenin and functions in AR mediated gene transcription. The identification and characterization of novel factors, such as CHD8, that can function as coactivators of AR mediated transcription could result in the discovery of novel diagnostic, preventative, or therapeutic targets for prostate cancer.

BODY

β-catenin, a known oncogene, functions as a transcriptional coactivator for AR through a direct protein/protein interaction (1-5). Although the significance of this interaction is not known, understanding the crosstalk between these oncogenic pathways is critical to delimitating the function of AR in tumorigenesis. To better understand the function of β-catenin in AR mediated transcription, we have identified novel chromatin remodeling enzyme, CHD8 that can associate with β-catenin and functions in AR mediated gene transcription. **The hypothesis of this proposal is that through a direct interaction with β-catenin, CHD8 is directed to androgen responsive promoters and functions in regulating transcription by altering the chromatin structure.** This hypothesis will be tested by addressing the following Aims: 1) Does β-catenin target CHD8 to AR responsive promoters? 2) Are CHD8 and β-catenin both required to affect AR transcriptional activity? 3) Is CHD8 required for prostate cancer progression? The proposed studies will provide detailed information concerning the role of β-catenin and CHD8 in AR mediated transcription. This information will be valuable for the understanding of how β-catenin mediates AR transcriptional activation, and also will provide general insight into the mechanisms of prostate neoplastic transformation through the control of chromatin structure.

The majority of the work proposed in months 1-12 addressed the first Aim by analyzing the interaction of CHD8 and β-catenin with the androgen receptor. Initial focus was placed on the production and optimization of recombinant proteins for protein/protein interaction studies (Task 1a). The recombinant proteins needed were CHD8, AR and β-catenin. As shown in the preliminary studies, full length CHD8 was successfully produced using a baculovirus expression system. Attempts were made at producing AR, but ultimately recombinant baculovirus for the expression of AR was obtained from Dr. James Dalton at Ohio State University (6). After significant optimization, expression conditions were identified for the production of full length 6-His tagged AR. Constructs were also generated for the production of full length β-catenin in a baculovirus expression system. Expression conditions were optimized, and full length β-catenin was also produced. A construct for the expression and purification of GST-tagged AR was obtained from the laboratory of our collaborator Dr. D. Robins. This construct was used as a

basis for the creation of various AR-GST fusions including a GST-N-terminal fragment, a GST-DBD-fragment, and a GST-LBD-fragment. Optimization and possible redesigning of these fragments is necessary as the GST-DBD-fragment fails to express detectable protein, and the GST-N-terminal fragment and GST-LBD-fragment produce multiple fragments that are essentially unusable in protein/protein interaction experiment. Several different fragments with alternate boundaries have been created and are currently being tested. The production of recombinant proteins must sometimes be designed and optimized empirically, and therefore can be slightly protracted.

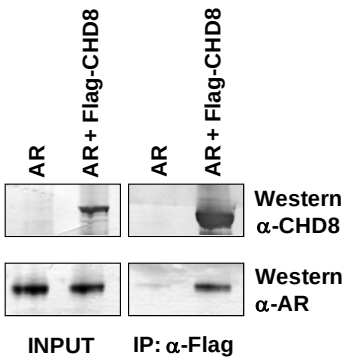

Figure 1. CHD8 directly interacts AR. SF9 cells were infected with recombinant baculovirus expressing AR or AR and Flag-CHD8. Lysates were prepared and immunoprecipitations were performed with Flag-M2 antibodies. After washing, purified samples were subjected to SDS-PAGE followed by Western blotting analysis using the antibodies indicated in the figure.

While specific fragments of AR are lacking, the generation of full length CHD8 and AR in a baculovirus expression system has allowed the test of a direct interaction between CHD8 and AR. This experiment is one of the main goals of Aim 1 (Task 1b). SF9 cells were infected with baculovirus expressing 6-His tagged AR in the presence or absence of baculovirus expressing Flag-tagged CHD8. Lysates were prepared from these infected cells and samples were then immunoprecipitated with anti-flag antibodies. As shown in Figure 1, only when cells are coinfected with Flag-tagged CHD8 can AR be immunoprecipitated with anti-flag antibodies. This result demonstrates a direct interaction between CHD8 and AR, and suggests that this interaction would not require β-catenin. As preliminary studies demonstrated a direct interaction between CHD8 and β-catenin, it is possible, however, to test for the creation of a tripartite complex between AR, CHD8 and β-catenin. In addition, once the GST-fragments of AR have been optimized and purified, it will be possible to test which domain of AR directly interacts with CHD8.

The second goal of Aim 1 was to extend the *in vitro* interaction studies to the description of the interaction between CHD8, AR, and β-catenin *in vivo* on chromatin using chromatin immunoprecipitation experiments (ChIPs). These experiments would benefit from the creation of stable β-catenin knock-down cells (Task1c). Constructs for the creation of these cell lines have been obtained from our consultant Dr. D. Turner. Several siRNA cassettes have been designed and tested. In order to quickly screen for constructs that effectively knockdown β-catenin activity, the following strategy was chosen: A colorectal cell line that has an activated Wnt signaling pathway (HCT116) was transfected with a reporter construct containing Tcf binding sites upstream of a luciferase reporter (TOP-Flash). In this cell line, robust reporter activity can be detected. These cells were also cotransfected with control siRNA constructs, or siRNA constructs targeting β-catenin. Of the transfected constructs, several displayed an appreciable knockdown of reporter activity, and the best construct was chosen for future experiments. Figure 2 shows the effect of this construct on reporter activity. For comparison,

cells were also transfected with a mutant form of Tcf4 that is incapable of binding β-catenin, and therefore acts as a dominant negative to inhibit the function of β-catenin. This data demonstrates that the constructed β-catenin siRNA construct effectively inhibits β-catenin mediated transcription, and that this inhibition is similar to levels produced when β-catenin can no longer interact with Tcf4 at the promoter. The β-catenin siRNA construct has been used in numerous attempts to create a stable LNCaP cell line. However, lines stabling expressing this construct have not been isolated. As outlined in the Contingencies section of Aim 1, an approach based on sorting transfected cells was then considered. siRNA vectors have been created that co-express the desired siRNA and also express a portion of the IL2 receptor (IL2Rα subunit). Transfected cells can then be separated using magnetic bead technology (anti-IL2Rα monoclonal antibody, Dynal Biotech). This approach now circumvents the requirement of created stable knockdown cells. The results of Task 1c have now suggested Task 2a should be accomplished using this same technology, and vectors required for this task are currently under production.

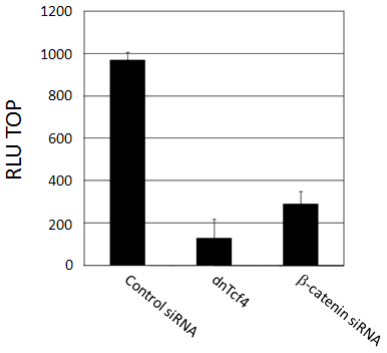

Figure 2. A β-catenin siRNA construct inhibits β-catenin mediated reporter activity. HCT116 cells were transfected the TOP-Flash luciferase reporter plasmid and control siRNA, β-catenin siRNA, or a dominant negative Tcf4. Shown is the relative luciferase units for each transfection condition.

Difficulties in creating stable cell lines have delayed the accomplishment of the second goal of Aim 1, and therefore Task 1d is still currently underway. Difficulties have also been encountered performing ChIP experiments with anti-β-catenin antibodies. Several different β-catenin antibodies have been tested for suitability in ChIP experiments, and these antibodies have failed to appreciable immunoprecipitate chromatin bound to β-catenin. Two recent studies have reported the identification by ChIP of β-catenin bound to the AR responsive PSA reporter (7,8). These studies used alternative antibodies that will be purchased and tested for functionality.

KEY RESEARCH ACCOMPLISHMENTS

- Recombinant proteins have been produced for the study of the interactions between CHD8, AR, and β-catenin (Task 1a). Production of several additional fragments is currently underway.
- Protein interaction studies have been performed to demonstrate the direct interaction of CHD8 and AR (Task 1b). This result will now direct research towards the investigation of a tripartite CHD8/AR/β-catenin complex.
- siRNA constructs have been made for the *in vivo* knockdown of β-catenin in stable cell lines.
- Creation of the above cell lines was not successful (Task 1c), and therefore, as outlined in the Contingency section, a system for sorting transfected cells has now been developed. These will be used in Task 1d and Task 2a.
- Chromatin immunoprecipitation experiments were commenced (Task 1d). However, two difficulties were encountered. These are currently being resolved using the above technique of cell sorting, and also by the purchase of β-catenin antibodies suitable for chromatin immunoprecipitation.

REPORTABLE OUTCOMES

The data presented here, along with the preliminary data, has been presented locally at the University of Michigan, both in the department, as well as interdepartmentally. In addition, this data has also been presented for an invited seminar at the University of Toledo, Department of Pharmacology.

CONCLUSION

As outlined in the original proposal, the majority of work proposed for Year 1 was centered on the production of recombinant proteins to investigate the association of CHD8, AR, and β-catenin. This work would then be extended to study their association *in vivo* at endogenous promoters. This work lays the foundation for the study of the function of CHD8 and β-catenin in AR mediated transcription (Year 2), with the ultimate goal being the understanding the role played by CHD8 in prostate cancer progression (Year 3). This understanding can lead to the development of chromatin remodeling inhibitors as therapeutics in prostate cancer.

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APPENDICES

None